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Early alterations on photosynthesis-related parameters in *Chlamydomonas reinhardtii* cells exposed to atrazine: A multiple approach study

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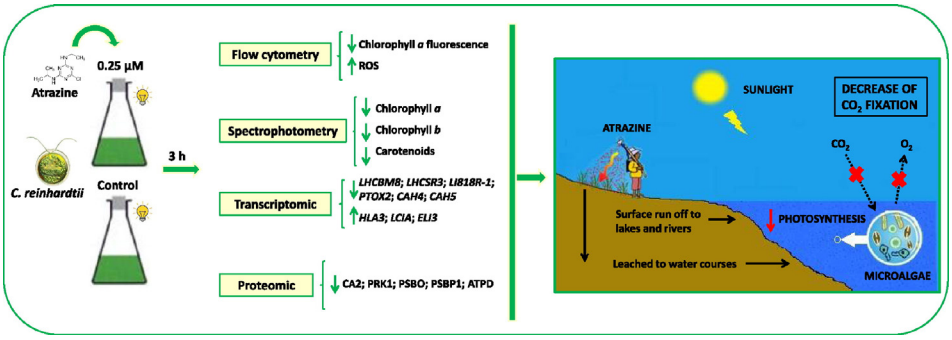
HIGHLIGHTS

- Pigment content and chlorophyll *a* fluorescence decreased in atrazine exposed cells.
- Atrazine induced an increase of reactive oxygen species level in treated cells.
- RNA-Seq analysis showed 9 differentially expressed photosynthesis-related genes.
- Proteomic analysis revealed changes in 5 proteins related to photosynthesis.

GRAPHICAL ABSTRACT



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ABSTRACT

Chlamydomonas reinhardtii cells were exposed to a sublethal concentration of the widespread herbicide atrazine for 3 h. Physiological cellular parameters, such as chlorophyll *a* fluorescence and oxidative stress monitored by flow cytometry and pigments levels were altered in microalgal cells exposed to 0.25 µM of atrazine. Furthermore, the effects of this herbicide on *C. reinhardtii* were explored using "omics" techniques. Transcriptomic analyses, carried out by RNA-Seq technique, displayed 9 differentially expressed genes, related to photosynthesis, between control cultures and atrazine exposed cultures. Proteomic profiles were obtained using iTRAQ tags and MALDI-MS/MS analysis, identifying important changes in the proteome during atrazine stress; 5 proteins related to photosynthesis were downexpressed. The results of these experiments advance the understanding of photosynthetic adjustments that occur during an early herbicide exposure. Inhibition of photosynthesis induced by atrazine toxicity will affect the entire physiological and biochemical states of microalgal cells.

Abbreviations: a.u., arbitrary units; CCM, carbon concentrating mechanism; Ci, inorganic carbon dioxide; CHAPS, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate; EB, etidium bromide; FCM, flow cytometry; FDR, false discovery rate; FS, forward scatter light; HE, hydroethidine; iTRAQ, isobaric tags for relative and absolute quantitation; LFC, $\log_2$ fold change; MALDI-TOF-MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; PSII, photosystem II; ROS, reactive oxygen species; RPM, reads per million mapped reads; SS, side scatter light; TCA, tricarboxylic acid cycle.

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1. Introduction

The occurrence of anthropogenic stress-induced chemicals, such as herbicides, in the aquatic environment presents a serious problem. These man-made organic toxicants are released into the aquatic environment and can affect non-target species. One of the herbicides most extensively applied in agriculture all over the world is atrazine. Its widespread application, persistence, and mobility have led to its frequent detection in ground and surface water sources (Hayes et al., 2010) and to list it as priority substance under the European Water Framework Directive as described in Directive 2013/39/EC (European Council, 2013). Atrazine inhibits photosynthesis blocking the photosynthetic electron transport at photosystem II (Rutherford and Krieger-Liszkay, 2001) and thereby energy production drops, preventing CO_2 fixation in target and non-target organisms. Atrazine-induced detrimental effects on the aquatic ecosystem and alterations in aquatic community structure have been reported previously (Choi et al., 2012; Didur et al., 2012; Sjollema et al., 2014; Weiner et al., 2004).

Microalgae have been recommended as test organisms in ecotoxicological studies because of their ecological relevance and sensitivity (Ma et al., 2006). All primary producers are the basis of the aquatic food web; therefore it is an interesting study of their photosynthetic state since any disturbance in productivity of the microalgae community can induce direct structural changes in the rest of the ecosystem (Campanella et al., 2001; Martinez et al., 2014; Rioboo et al., 2007). Regarding toxicity investigations and risk assessment with microalgae, integral endpoints, such as growth and reproduction, are traditionally monitored. However, before these endpoints show a significant change, the exposure may affect other cellular physiological parameters at much lower toxicant concentrations (Nestler et al., 2012).

The photosynthetic state of microalgae can be measured by quantifying the pigment content, which has been used as biomarker of exposure to herbicides in algae (Couderchet and Vernet, 2003). Fluorescence measurements have also been proposed as simple, rapid and sensitive methods to detect the photoinhibitory effects of environmental stressors on phytoplankton (Geoffroy et al., 2007; Juneau et al., 2002). Flow cytometry (FCM) is an alternative to the standard algal population based endpoints, since it allows the characterization of the microalgal response at a single-cell level close to *in vivo* conditions. The *in vivo* chlorophyll *a* fluorescence of green algae can be used as a tool to detect negative alterations on photosynthesis (Bi Fai et al., 2007; Chalifour et al., 2009; Cid et al., 1995; Ekelund and Aronson, 2007; González-Barreiro et al., 2004; Prado et al., 2011).

Currently, with the development of the omics, new alternatives arise to study the effects of pollutants on microalgae (Dowling and Sheehan, 2006; Esperanza et al., 2015; Jamers et al., 2009; Monsinjon and Knigge, 2007). Transcriptomics and proteomics can be used to detect and characterize responses to external stimulus, and have a great potential for investigating stress mechanisms and responses affecting growth and other physiological and biochemical endpoints (Jamers and de Coen, 2010; Nestler et al., 2012; Subramanian et al., 2014), being increasingly applied in ecotoxicology and other fields of biological science. The availability of the sequenced genome of the microalgal species *Chlamydomonas reinhardtii*, with adequate annotation and metabolic pathway information, facilitates high-throughput analyses of transcriptional and proteomic profiling (Merchant et al., 2007).

Previous research has demonstrated that *C. reinhardtii* cells exposed to atrazine change their metabolism and get energy mainly by heterotrophic pathways (Esperanza et al., 2015). In a global climate change scenario with a dramatic increase in CO_2 levels, it is very important to know the behavior of carbon fixing organisms. Thus, the aim of the present study was to determine alterations in parameters directly or indirectly related to photosynthesis after only 3 h of exposure to the herbicide. For this purpose, different methodological approaches were applied. First, a traditional spectrophotometric method for the quantification of pigments content was carried out. Additionally, flow cytometry was used to determine chlorophyll *a* fluorescence and reactive oxygen species level (ROS). Moreover, unlike standard ecotoxicity studies, detailed quantitative transcriptomic profiling of algal cells exposed to atrazine compared with those cultured without herbicide was assessed, looking for changes in the regulation of transcription. Furthermore, a proteomic analysis was carried out to observe early protein alterations due to the stress directly caused by the herbicide.

2. Materials and methods

2.1. Microalgal cultures

The unicellular green alga *C. reinhardtii* Dangeard (strain CCAP 11/32A mt +) was obtained from the Culture Collection of Algae and Protozoa of Dunstaffnage Marine Laboratory (Scotland, UK). *C. reinhardtii* cells were cultured in Tris-minimal phosphate medium (Harris, 1989) on a rotary shaker set at 150 rpm, under controlled conditions: 22 ± 1 °C and illuminated with 100 μmol photon m^{-2} s^{-1} under a 12:12 h light:dark cycle. Cells in mid-logarithmic growth phase were used as inoculum for the different assays. Initial cell density for each experiment was 2×10^5 cells mL^{-1}.

Before each experiment, fresh stock solutions of atrazine were prepared by dissolving the pure compound (Sigma-Aldrich, MW: 215.68) in methanol and filtering through 0.2 μm membrane filters. Also control cultures were included, to which only methanol was added. No significant differences between nominal and effective concentration of atrazine were found using a gas chromatography/mass spectrometry analysis.

All cultures were set up in triplicate for 3 h and at least two independent experiments were carried out for each parameter analyzed. This time point was selected considering previous cytometry studies where the effects of atrazine were analyzed every hour during 24 h and changes in the cellular metabolic activity and ROS formation were detected after 3 h of atrazine exposure (unpublished data).

2.2. Growth measurement

A growth inhibition test for *C. reinhardtii* using atrazine concentrations ranged from 0.1 to 2 μM was carried out to determine the herbicide concentration used for the following determinations of the present study. Cell density was daily determined for 96 h by counting culture aliquots in the flow cytometer. For absolute cell counting, a suspension of fluorescent polystyrene microspheres (Flow-Count Fluorospheres; Beckman Coulter) with known concentration was added as an internal reference to all cell samples. Growth rates (μ) expressed as day^{-1} were calculated *via* the formula $\mu = [\ln(N_t) - \ln(N_0)] / \ln 2(t - t_0)$ where N_t is the cell density at time t and N_0 is the cell density at time 0. The 96 h EC_{50} value for growth was calculated, based on growth rate data, using the computer program CompuSyn (Chou and Martin, 2005).

2.3. Photosynthetic pigment content

Pigments were extracted from a concentrated algal sample in a 90% acetone aqueous solution and determined by measuring the absorbance of the extract using a Shimadzu UV-1700 spectrophotometer at appropriate wavelengths (664, 647 and 480 nm). The resulting absorbance measurements were translated to chlorophylls and carotenoids according to Jeffrey and Humphrey (1975) and Strickland and Parsons (1972), respectively. The equations used to calculate the pigment concentrations in the extract are:

Chlorophyll $a = 11.93\ A664 - 1.93\ A647$

Chlorophyll $b = 20.36\ A647 - 5.50\ A664$

Carotenoids $= 4.0\ A480$